Can Alpha-1-Protease Inhibitor Be Used in Replacement Therapy?[1-3]

CHARLES GLASER

SUMMARY We propose that alpha-1-protease inhibitor (α_1PI) can assume a major and beneficial role in preventing emphysema in α_1PI-deficient individuals, and may also prove of value in the treatment of adult respiratory distress syndrome (ARDS). α_1PI has a single, unusual disulfide bond that consists of a cysteine residue in the peptide chain covalently bound to a free amino acid cysteine. The linkage can be broken by reductants without adversely affecting the stability or the inhibitory activities of the protein. As a result of this property, α_1PI can be effectively separated in solution from many other plasma proteins by salting out the contaminants in the presence of strong reductants. We have applied the technique of reductive-salting out, coupled with more conventional DEAE-anion exchange chromatography to isolate α_1PI from Cohn Fraction IV-1, a relatively unused side product in the worldwide production of albumin and immunoglobulins. Furthermore, we have demonstrated that the product can be effectively pasteurized in the presence of various stabilizing additives. The necessary ingredients now exist for extensive clinical studies in the years ahead.

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Introduction

In this report, I will discuss the biology and chemistry of alpha-1-protease inhibitor (α_1PI, also called alpha-1-antitrypsin) in order to express the variables that must be considered to permit its use as a therapeutic agent.

First, the genetics of α_1PI will be presented as related to the problem at hand. In the discussion of its biology, the distribution, production, and clearance of α_1PI will be reviewed. Clinical states with apparent deficiencies of α_1PI will be listed, and our new method for isolation of α_1PI, which is compatible with the requirements of industrial-level plasma fractionation, will be discussed.

Finally, the pitfalls of employing such therapy will be described and alternatives will be briefly alluded to.

Genetic and Biologic Considerations

Proteolytic enzyme inhibitors are of fundamental importance in the regulation of key physiologic processes, including blood clotting and the dissolution of clots, blood pressure, hormone action, tissue remodeling and wound healing, the immune system, and various cellular processes including differentiation, division, recognition, and transformation (1-6). In plasma these inhibitors comprise about 10% of the total protein and include α_1PI (also called alpha-1-antitrypsin), α_2-macroglobulin, antithrombin III, α_1-antichymotrypsin, α_2-plasmin inhibitor, C_1-inactivator, and inter-alpha trypsin inhibitor.

α_1PI is the most prevalent of the protease plasma inhibitors. It is a glycoprotein of molecular weight ~ 50,000, and is also present in interstitial fluids, lymph, genital secretions, tears, saliva, nasal and pulmonary secretions, cerebrospinal and amniotic fluids, colostrum, platelets, and megakaryocytes. α_1PI is synthesized in liver parenchymal cells (7). It is secreted into the circulation in multiple forms as visualized by discontinuous starch gel electrophoresis (8) or isoelectric focusing (9), where as many as 8 discrete bands are seen. The gene locus is named Pi (for protease inhibitor) and the more than 20 genetic variants are given letter designations Pi B, C, D. . . M. . . Z, etc., according to their electrophoretic mobility. These alleles are expressed in an autosomal codominant fashion. The PiM allele is most common with a gene frequency of ~0.9, and individuals homozygous for this allele have ~1.3 mg α_1PI/ml of plasma (10).

Several of the variant forms of α_1PI are associated with decreased levels of circulating inhibitor. These include the alleles PiZ and PiS, as well as the less common Pi (null gene) (11), which is associated with little or no detectable product.

The pathophysiologic significance of low circulating levels of α_1PI derives from the strong association of pulmonary emphysema with the homozygous Z allele (12), and this can result from improperly regulated proteases released from inflammatory cells. Studies on the catabolism of PiM and PiZ forms of α_1PI indicate that both proteins have similar half-lives, about 5 days for the Z-protein (13), and 6 to 7 days for the M-protein (13, 14). Clearance from plasma for both forms of α_1PI is dramatically enhanced upon the loss of terminal sialic acid residues (15, 16), and this is apparently the result of affinity by the newly exposed galactosyl residues for a liver membrane receptor (15). Detailed investigation on a whole series of plasma glycoproteins indicates that the hepatic recognition and binding of sialic acid deficient molecules is a signal for transport to liver lysosomes where proteolytic breakdown occurs (17).

Feasibility of Replacement of α_1PI Deficient Subjects

As shown by Gadek *et al*, individuals who are homozygous for the deficiency gene Z have no effective antiprotease shield in their lower respiratory tract (18). α_2-macroglobulin, a large serum antielastase, and the bronchial mucus inhibitor, an antielastase of the central airways, do not protect the alveolar structure. In a follow-up study, α_1PI of ~ 17.5% purity was obtained from normal donors (Pi type MM) by fractional precipitations with polyethylene glycol (19). Five emphysematous individuals with severe α_1PI deficiency (Pi type ZZ) received weekly injections of 4 grams of α_1PI intravenously for 4 weeks. The treatment resulted in an elevation of serum α_1PI levels to ≥ 35% of normal (i.e., > 70 mg/dl at all times), and analysis of bronchoalveolar lavage fluid indicated that an effective antielastase activity was established within the alveolar structures. No problems were encountered with sterility, pyrogenicity, or hepatitis. Also, none of the patients showed any antibody formation against PiM protein up to one year following infusion. Finally, no signs of allergic reaction or volume overload were found. These results are very encouraging, although the introduction of α_1PI at the low level of purity used by the authors is impractical for long-term treatment, because the infusion of such a protein load (22.9 g/285ml/week) required 6 to 8 hours.

Would α_1PI be a Useful Therapeutic Agent in Other Diseases?

It has been demonstrated that unrestrained proteolysis occurs in the lungs in patients with adult respiratory distress syndrome (See Cochrane *et al*, this symposium). In addition, active neutrophil proteases have been demonstrated in purulent secretions of patients with chronic bronchitis (20), in patients with pneumonia (see Cochrane *et al* and Lonky, this symposium) and free proteases have been suggested as having a pathogenetic role in pancreatitis. In addition, investigators have not determined whether the liver diseases seen in infants with PiZ phenotype might be favorably af-

[1] From the Medical Research Institute of San Francisco, San Francisco, California.

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[3] Requests for reprints should be addressed to Dr. Charles B. Glaser, Medical Research Institute of San Francisco, 2200 Webster Street, Room 427, San Francisco, California 94155.

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TABLE 1

PURIFICATION OF α_1-PROTEASE INHIBITOR FROM COHN IV-1 BY CONVENTIONAL MEANS*

	Protein (mg)†	α_1-PI (mg)‡	Specific TIC (mg/mg)†§	Activity Recovered (%)‖	Purification Factor
Cohn fraction IV-1	14,000	1,490	0.059	100	1.0
DEAE-cellulose	2,240	1,020	0.18	68.5	3.1
QAE-sephadex	621	690	0.65	46.3	11.0
Concanavalin A	526	650	0.70	43.6	11.9
G-150 Sephadex	401	452	0.86 (0.85)¶	30.2	14.8

* Taken from reference 23.
† Determined by the method of Lowry, *et al*, (52).
‡ Determined by radial immunodiffusion and expressed as total mgs of antigen present in the fraction.
§ Trypsin inhibitory capacity divided by the protein concentration of the fraction in mg/ml.
‖ Based on the radial immunodiffusion assay.
¶ Based on $E^{0.1\%}_{1\ cm,\ 280\ nm} = 0.50$.

fected by raising plasma α_1PI levels to normal. Therefore α_1PI may have potential as a therapeutic agent for diseases other than emphysema.

Isolation of α_1PI on an Industrial Scale

α_1PI has been isolated in many laboratories as a relatively homogenous, stable, and active product suitable for research purposes (21–25). In deriving a product suitable for large-scale clinical application, however, many additional considerations become important including the source of starting material, the safety of each separation step employed, the feasibility of shifting each technique to large-scale processing, the reproducibility, and the cost. In addition, the product derived should be biologically potent, sterile, and safe (i.e., free of toxins, pyrogens, immunogens, carcinogens, teratogens, materials affecting blood pressure, and hepatitis). Beginning in 1974, we undertook the task of trying to overcome the various obstacles in providing sufficient α_1PI to the medical community for its clinical needs. The following is a report of our progress to date.

The most common large-scale plasma fractionation scheme in use today throughout the world continues to be the cold ethanol fractionation procedure of Cohn *et al* (i.e., Method 6) (26), or modifications thereof (27). In 1974, we received from Cutter Laboratories (Berkeley, CA) several Cohn Fractions for evaluation including II + III, IV-1, and IV-4. Each was provided as a frozen, homogenized paste. A preliminary evaluation revealed that IV-1 was most suitable for our purposes. This relatively unused byproduct is recovered during albumin and immunoglobulin production. It is formed as a precipitate in the fractionation process at ~ pH 5.2 and ~ 20% ethanol, and typically contains at least the following proteins: α_1PI, albumin, antichymotrypsin, antithrombin III, α-acid glycoprotein, ceruloplasmin, transferrin, α_1-lipoprotein, haptoglobin, inter-α-trypsin inhibitor, C_1-inactivator, α_2-macroglobulin, IgG, and IgA.

Approximately 10% of the protein present in the Cohn IV-1 paste is α_1PI as assayed by radialimmunodiffusion, and this corresponds to ~ 30% of the α_1PI in plasma. The distribution of α_1PI in other Cohn Fractions has not been described.

Our original purification scheme (23) involved activation of the inhibitor in slightly basic media, and successive column chromatographic steps on DEAE-cellulose, QAE Sephadex, Sepharose-bound Concanavalin A, and Sephadex G-150, and demonstrated the feasibility of isolating α_1PI by a method that could be directly applied to worldwide plasma fractionation schemes (table 1). It indicated in particular that most of the α_1PI that had been treated at pH 5.2 was not permanently inactivated, although later work (unpublished) in our laboratory shows that, for some lots of Cohn IV-I, as much as 30% of the α_1PI is in an aggregate form that is irreversibly inactivated.

The finding that α_1PI could be recovered in active form from low pH solutions was in contrast to the reports of others who claimed that low pH conditions would permanently inactivate α_1PI. To investigate this matter in more detail, we initiated a study of the low pH inactivation of α_1PI (28). Our investigation indicated that active α_1PI could be stored at pH 2–4 at 4° C for variable time periods with eventual recovery of considerable activity against trypsin and elastase. The inactivation by acid apparently occurs by two means:

(*1*) A rapid and largely reversible conformational change in the monomeric form of α_1PI that is accompanied by nearly complete loss of enzyme inhibitory properties and a large increase in the tryptophan fluorescence emission at 335 nm (figure 1). Reincubation of protein in basic media results in substantial recovery of activity within 1 h and this is preceded by a time-dependent decrease in the fluorescence measured at 335 nm. Almost all of the regenerated biological activity resides in the monomeric fraction that regains 91 to 98% of its original trypsin inhibitory capacity.

(*2*) The slow formation of higher molecular weight aggregates of α_1PI that have little or no enzyme inhibitory properties, and are not readily dissociated (table 2).

Although the isolation of α_1PI from Cohn IV-1 indicated promise, in practical terms it did not meet the necessary criteria for producing a useful blood product. The use of Sepharose-bound Concanavalin A is unacceptable, as the leakage of even minute amounts of Con A into the media could introduce the serious risk of a major antigenic reaction in the recipient. In addition, although the gel filtration step is safe and reproducible, it is not an approach that can be used for purification of greater than several grams at a time, and therefore is not practical.

Concurrently, we investigated the chem-

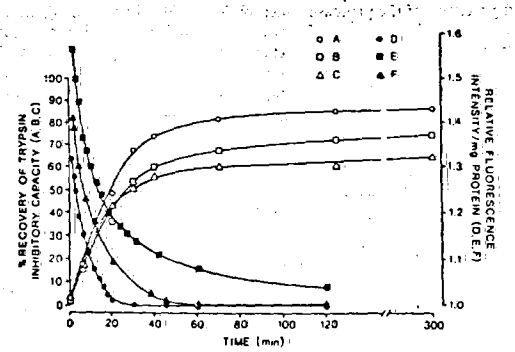

Fig. 1. The increase in trypsin inhibitory capacity (A, B, C) and corresponding decrease in the fluorescence intensity (D, E, F) as a function of time following titration of various acidic solutions to pH 8.0–8.2 with an equal volume of 0.66 M Tris buffer, pH 8.5. The original solutions were as follows: A, D (0.1 M glycine/HCl, pH 3); C, F(0.1 M glycine/HCl, pH 2); B, E(0.1 M ammonium acetate, pH 4). The acid incubation period was 30 min. The recorded fluorescence intensity in each case is relative to that of a control solution prepared by dissolving α_1PI in a pH 8.0–8.2 buffered solution consisting of one part of the appropriate low pH solution and one part of 0.66 M Tris HCl, pH 8.5. Both the trypsin inhibitory capacity (defined as 100%) and the relative fluorescence intensity (defined as 1.0) remained constant during the time course of the experiment when measured using a control α_1PI.

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TABLE 2

PERCENT TRYPSIN INHIBITORY CAPACITY RECOVERED FROM SEPHADEX G-150 COLUMNS AFTER LOW pH INCUBATION*

	Monomer		Polymer	
	30 min	48 h	30 min	48 h
0.1 M glycine/HCl buffer, pH 2.0	98† (63)‡	72 (46)	< 2	< 10
0.1 M glycine/HCl buffer, pH 3.0	93 (75)	94 (58)	< 2	< 10
0.1 M ammonium acetate, pH 4.0	91 (78)	84 (36)	< 2	< 10

* Taken from reference 28; following incubation at low pH for 30 min or 48 h, solutions were dialyzed to pH 8.0 for 24 h.
† % of trypsin inhibitory capacity recovered relative to control solution of α_1PI that has not undergone acid treatment.
‡ Figures in parentheses represent the % of α_1PI recovered in the monomer fractions based on $E^{0.1\%}_{1\text{ cm},280\text{ nm}} = 0.50$.

istry of the disulfide bond of α_1PI (29). α_1PI has a single, unusual disulfide bond that consists of a cysteine containing region (i.e., Phe-Asn-Ile-Gln-His-Cys-Lys) within the polypeptide chain which is covalently linked to free cysteine or glutathione via a disulfide bridge. When this disulfide bridge was broken by reduction and then alkylated with iodoacetamide, the resultant product was found to be fully functional as an inhibitor of trypsin and elastase in esterolytic and proteolytic assays. The modified protein was also found to be identical to native α_1PI when analyzed by immunologic, electrophoretic, and spectral methods.

These findings concerning the disulfide bond of α_1PI prompted us to explore a new approach to the purification. We treated a mixture of plasma proteins containing α_1PI with a strong reductant (e.g., dithiothreitol, DTT). The disulfide bridge in α_1PI should be reduced along with large numbers of disulfide bonds in many of the other proteins in the media. Because it is well known that S-S bridges help to stabilize proteins in solution, many if not most of the undesired proteins should be destabilized by this reductive treatment and made more amenable to precipitation [e.g., in $(NH_4)_2SO_4$ or polyethylene glycol]. We had earlier determined that we could effectively remove ~ 70% of the contaminating protein with a recovery of 70 to 75% of α_1PI by salting out the reactivated Cohn IV-1 mixture in 50% $(NH_4)_2SO_4$. We used this condition to test the effect of treatment of the Cohn IV-1 media with various concentrations of DTT at 25° C for 24 h before salting out at 4° C with $(NH_4)_2SO_4$, stirring overnight and centrifuging. In addition, in some experiments, we preincubated the media with 2.5% by weight of Aerosil 380 (Degussa, Inc., Teterboro, NJ), a fumed silica product that selectively adsorbs several plasma proteins including α- and β-lipoproteins and plasminogen (30, 31).

The reductive salting out technique is highly effective at concentrations of 0.025 M DTT or higher and the addition of Aerosil to the media gives an additional purification, as illustrated in figure 2. Centrifugation of the Aerosil-DTT-$(NH_4)_2SO_4$-treated Cohn IV-1 solution effectively removes ~ 95% of the overall protein from the media (figure 2B), giving a purification factor of nearly 14 compared with ~ 8 for product obtained after treatment with DTT and $(NH_4)_2SO_4$ but no Aerosil, and only 3 for product obtained by conventional salting out (~ 50% $(NH_4)_2SO_4$) (figure 2A). Albumin, which is present in ~ 15% in the Cohn IV-1 media, is completely removed by the reductive treatment with or without Aerosil (figure 2C). Approximately 70 to 75% of the α_1PI was recovered from the treatment in all cases (figure 2D). Similar matrices were conducted to optimize pH, temperature, and incubation time of the reducing step (data not shown). Neither cysteine nor mercaptoethanol at concentrations as high as 1.0 M could approximate the results found with DTT, indicating the

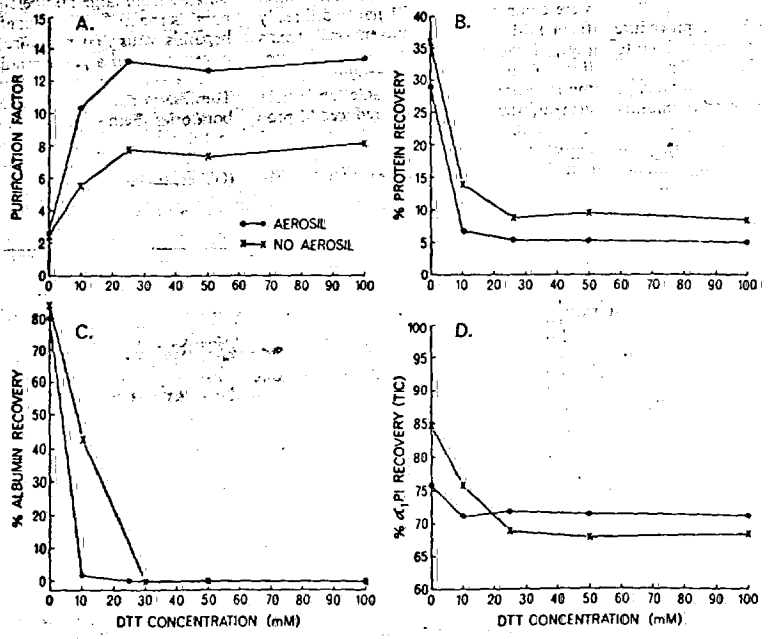

Fig. 2. Purification profile of α_1PI from Cohn IV-1 as a function of dithiothreitol concentration. A. Purification factor; B. Percent total protein recovery; C. Percent albumin recovery; D. Percent α_1PI recovery measured by TIC.

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special importance of this reducing agent that results from its ability to form a stable 5-membered ring on oxidation, shifting the equilibrium toward a reduced product.

When we had determined the proper conditions, Cohn IV-1 paste was activated, treated with Aerosil and DTT, and salted out with $(NH_4)_2SO_4$ according to the following scheme (32):

50 g of Cohn IV-1 (15 g protein, 1 g active α_1PI) dissolved in 0.5 L of 0.1 M Tris-HCl, pH 8.5, 24 h, 4° C.

2.5% dry Aerosil (final concentration), followed by 0.025 M DTT, stir for 24 h at 25° C.

50% saturated $(NH_4)_2SO_4$, final concentration, stir 16 h at 4° C.

Spin (5,000 × *g*, 30 min), wash, spin, concentrate to 300 ml by ultrafiltration (10,000 M.W. cut off).

Dialyze against 4L 0.01 M cysteine, 0.01 M phosphate (pH 7.6) at 25° C, then phosphate buffer alone.

The dialysate was then loaded onto a DEAE-cellulose column equilibrated with 10mM sodium phosphate (pH 7.6) to a concentration of 40–75 mg protein/ml of resin. The column was washed with equilibrating buffer until the $OD_{280\,nm}$ approached zero, and then α_1PI was eluted with a gradient of 10 mM phosphate (pH 7.6) to 10 mM phosphate (pH 6.0), 0.1 M NaCl. Fractions having a specific activity of > 0.90 mg/mg were pooled, dialyzed against 10 mM NH_4HCO_3, and lyophilized (figure 3).

As indicated in table 3, the procedure yields a highly purified product with 40 to 45% recovery. Analysis of the final product using radial immunodiffusion against a large number of specific antibodies to plasma proteins reveals only antithrombin III as an impurity (< 1%). In several selected experiments, a specific elastase inhibiting activity of ~ 99% is found, compared with a standard α_1PI preparation (MM type).

Figure 4 shows an SDS-polyacrylamide gel electrophoresis pattern of Cohn IV-1 (4B) and of several partially purified fractions derived from salting-out (with and without Aerosil and/or DTT [4C, 4E, and 4F], and following DEAE-cellulose chromatography [4G]. The product obtained by Aerosil-DTT-$(NH_4)_2SO_4$ treatment (4E) compared with conventional salting out (no Aerosil, no DTT) (4C), or to salting out in the presence of Aerosil (4D), indicates the effectiveness of DTT in the isolation scheme. The ability of the Aerosil to remove most of the lower molecular weight impurities is seen in 4E (with Aerosil) versus 4F (without Aerosil), and is important as these bands are sometimes associated with blood-pressure regulating substances. Aerosil effectively removes all lipoprotein at the reductive-salting-out step and additionally improves the removal of ceruloplasmin and transferrin, as shown by radialimmunodiffusion. The final product (4G), obtained following the DEAE-chromatography, appears homogeneous and migrates with the same mobility as a control sample of M-protein purified from plasma by conventional means (4H).

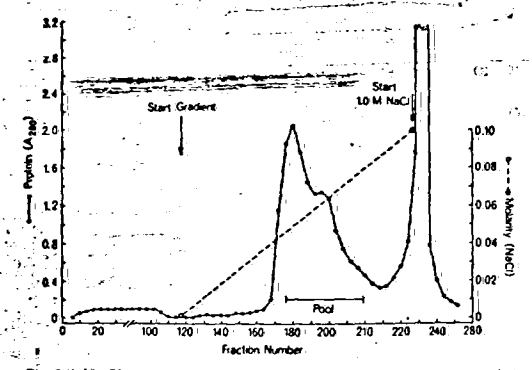
To evaluate whether cysteine dialysis results in the reforming of the α_1PI disulfide bridge in the final product, the extent of incorporation of ^{14}C-containing iodoacetamide was measured for both reduced and nonreduced protein, and the results also were compared with α_1PI purified directly from PiM plasma by conventional means (i.e., without reductive-salting out). The reduced Cohn IV-1 derived product showed the incorporation of 0.80 mole label/mole protein, whereas that of the reduced M-protein was 0.92 mole/mole. In the absence of reductant, the Cohn product showed 0.06 mole/mole incorporation whereas the M-protein showed 0.02 mole/mole. These results indicate that the α_1PI derived by the reductive-salting out method has a protected thiol function (i.e., nonreactive in the absence of reductants), presumably in the form of disulfide bond with cysteine, since little or no dimer or aggregate is seen.

Phenotyping of the product gave a pattern indistinguishable from the Pi-type M product. This is not a surprising result for a pooled plasma pattern because of the high gene frequency of the M-protein.

The LD_{50} in mice and the blood pressure effects in anesthetized rats were used to compare α_1PI prepared in Cutter Laboratories by a procedure similar to our own to α_1PI purified by more conventional procedures. The LD_{50} indicated a low toxicity. In rats the more pure α_1PI preparations (both DTT-treated and conventional) were nonhypertensive and the functional elastase inhibitor capacity and antigenic half-lives of all preparations were similar (D. Schroeder, Cutter Laboratories, private communication).

The reductive-salting out is applicable to other fluids and this is illustrated in table 4 for diluted human plasma. A purification factor of ~ 11 was achieved in the presence of reductant whereas no purification was found in its absence (no Aerosil was used in these experiments).

For long-term treatment of α_1PI-deficient individuals, a major limitation to the use of this inhibitor, or any other plasma product isolated from large numbers of human donors, is the high risk of contamination by hepatitis virus. To minimize this risk, we have developed a pasteurization procedure in collaboration with Drs. Ken Ingham and Tom Busby at the American Red Cross Laboratories, Bethesda, MD. A series of thermal denaturation profiles of α_1PI was obtained using various stabilizing molecules (Glaser, Ingham, unpublished observations).



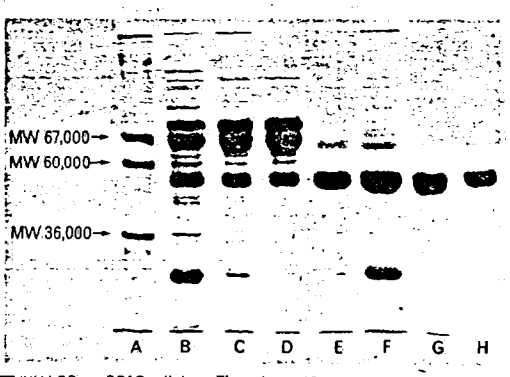


Fig. 3 (*left*). Chromatography of α_1PI obtained from purification step with Aerosil-DTT-$(NH_4)_2SO_4$ on DEAE-cellulose. The column (13 × 1.5 cm) was equilibrated with 0.01 M sodium phosphate (pH 7.6). After application of the sample, the column was washed with equilibration buffer and a linear gradient to 0.01 M sodium phosphate + 0.1 M NaCl, pH 6.0, was initiated at fraction 118 (500 ml total). Fig. 4 (*right*). Gel slab electrophoresis of samples from successive steps in the purification of α_1PI from Cohn IV-1. A. M.W. standards (Pharmacia); B. Cohn IV-1 (40 μg); C. Supernatant after 50% $(NH_4)_2SO_4$ precipitation (30 μg); D. Supernatant after 50% $(NH_4)_2SO_4$ and 2.5% Aerosil (30 μg); E. as in D, plus 25mM DTT (30 μg); F. 50% $(NH_4)_2SO_4$ and 25 mM DTT (30 μg); G. α_1PI from Cohn IV-1 after DEAE cellulose chromatography (20 μg); H. α_1PI (Pi type MM) isolated from human serum by conventional methods (20 μg).

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TABLE 3

PURIFICATION OF α_1-PROTEASE INHIBITOR BY THE REDUCTIVE-SALTING OUT METHOD*

	Protein mg	α_1PI Corr (TIC)	Specific Activity mg active α_1PI / mg total protein	α_1PI Yield %	Purification Factor
Cohn IV-I	16,050†	900	0.056	—	—
Aerosil + dithiothreitol + $(NH_4)_2SO_4$	770†	531	0.692	59	12.4
DEAE-cellulose	391‡	387	0.990	43	18.9

* Taken from reference 32.
† Determined by Lowry with a BSA standard.
‡ Determined by using $E^{0.1\%,\ 1\ cm}_{280\ nm} = 0.50$.

The increase in 1,8-anilinonaphthalene sulfonate (ANS) binding as a consequence of denaturation was used to monitor each experiment (33).

Solvolytic conditions that resulted in substantial shifts to higher temperatures in the ANS binding curves, especially where the onset of denaturation was above 60° C, were considered for further evaluation. Under selected solvolytic conditions, α_1PI was heated for 10 hours at 60° C and its activity was measured. Pasteurization in the presence of combinations of citrate (1.2M) and either EDTA (0.5 M) or gluconate (1.2 M) results in a product that is 90 to 95% active against trypsin and elastase. Aggregation accounts for some, but not all, of the activity loss. Loss of activity is not affected by protein concentration. Reductant or free radical scavengers in the media (mercaptoethanol, ascorbic acid, 2,6-di-tertbutyl-4-methylphenol [BHT]) do not increase the yield of active product.

In conclusion, our work has shown that α_1PI can be obtained as a blood product by means that are inexpensive, simple, safe, and reproducible, and which give a relatively pure product (> 90%) in high yield by procedures that are applicable to large scale industrial processing. Although we carried out the DEAE-cellulose separation via column chromatography, batch-mode processing should certainly be achievable although some loss of purity would be expected. In addition, we have developed a pasteurization protocol that results in the preservation of 90 to 95% of α_1PI activity following heat treatment at 60° C for 10 hours.

Potential Hazards of α_1PI Replacement Therapy

Because the consequences of a severe deficiency of α_1PI are pulmonary emphysema in ~ 80% of the affected individuals, then a strong logic exists for prevention of this disorder with α_1PI replacement therapy. In this regard, it is appropriate to compare the structure of the M protein to its Z variant.

An amino acid substitution of glutamic acid in the normal protein to lysine in the variant protein was found by peptide fingerprinting (34, 35). A second substitution of glutamic acid to glutamine also was reported by one of the groups (35). In addition, the Z protein may contain less carbohydrate than the M protein. In one study, a possible sugar difference was found for residues of sialic acid, the terminal sugar in most plasma glycoproteins (36), and in other reports the Z protein also was found to be deficient in glucosamine and the neutral sugars mannose and galactose, as well as sialic acid (37, 38). In experiments from this laboratory involving successive treatment with neuraminidase and galactose oxidase, followed by reduction with tritium-labeled sodium borohydride and isolation of the neutral sugar fraction, it was shown that the Z protein has a diminished content of both galactose and sialic acid relative to the M form, and additional experiments indicate that neither protein contains terminal galactosyl residues (15). The question of carbohydrate differences is still unresolved, however, because a recent investigation revealed no carbohydrate differences between the two forms (39).

The two proteins were compared by circular dichroism spectroscopy and by fluorescence quenching experiments using anionic (I^-) and neutral (i.e., acrylamide) probes (40). Both proteins share a similar secondary structure, i.e., ~ 45 to 50% α-helix and 15 to 20% β-structure. Evidence was accumulated to show that the microenvironment in the vicinity of the 3 tryptophanyl residues is altered in Z form compared with the M form as shown by (*a*) the absence of the positive dichroic band in the 290 to 300 nm region of the circular dichroism spectra, (*b*) a > 50 % increase in quantum yield in the tryptophanyl fluorescence emission spectra, (*c*) an increased accessibility of tryptophan to quenching by iodide, and (*d*) acrylamide quenching experiments which indicate that all tryptophanyl residues in the Z protein are quenched equally or that quenching is dominated by a single residue, whereas in the M protein, heterogeneous quenching occurs.

As discussed by Boyer *et al* (41), individuals with mutant genes that result in the absence of production of a particular protein are especially likely to be immunologically intolerant of such a protein. Thus, in terms of protein replacement, the presence of small levels of the protein native to the recipient is distinctly better than none. We can distinguish between cross-reactive negative (CRM−) mutants (i.e., those individuals with no recognizable version of a particular protein) and cross-reactive positive (CRM+) mutants at the same locus. Because most CRM+ mutants (PiM vs. PiZ) involve only one amino acid residue substitution out of hundreds, the risk of immunologic recognition of the altered sequence or of any consequent local conformational changes imposed by the sequence variation should, in general, be much less severe than in CRM− individuals. In CRM− individuals, all peptide regions and conformational arrays are potential antigenic sites. Also, it should be pointed out that some CRM− mutations may turn out to be CRM+ using more sensitive means of detection. Just such a finding appears likely for at least some cases attributed to the Pi-null gene, where an electrophoretic pattern identical to that of very dilute PiM serum could be produced by radioautography (42). Thus far, limited experience with the repeated infusion of M protein into Z recipients gives no indication that the M protein contains any novel antigenic sites compared with the Z-variant. It should be noted that a possible antigenic reaction is a legitimate concern for long-term replacement therapy in α_1PI-deficient individuals, but it is not an important issue for potential treatment of ARDS.

Of course, many questions remain concerning the use of α_1PI in replacement ther-

TABLE 4

THE PARTIAL PURIFICATION OF α_1-PROTEASE INHIBITOR FROM PLASMA BY REDUCTIVE-SALTING OUT*

0.1 M dithiothreitol	Dilution	Protein† Initial	Protein† Final	% Recovery (Protein)	% Recovery of α_1PI‡	Purification Factor
+	2-fold	501.0	46.2	9	61	6.66
+	4-fold	250.5	17.9	7	68	9.60
+	6-fold	167.0	10.6	6.3	72	11.40
−	2-fold	501.0	307.8	61.0	65	1.07
−	4-fold	250.5	161.0	64.0	78	1.22
−	6-fold	167	121.6	73.0	81	1.12

* Taken from reference 32.
† Determined by Lowry.
‡ Determined by trypsin inhibitory capacity.

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apy. These include the conditions of storage, the concentration of protein, the degree of purity, the pH, and the infusion rate. One must carefully monitor blood pressure effects that could be introduced by minor contaminants from the kallikrein-kinin system (43, 44). One also must consider the possibility that any aggregates formed on storage or heating of the protein will introduce new antigenic determinants in the system as have been reported for albumin (43, 45) and immunoglobulin (44). In the studies of Gadek *et al* (18), the concentration of total protein employed was ~ 8.0 g/100 ml and the osmolarity of the preparation was 450 mosmol/l. If an individual required 4.0 g of α_1PI/ week and the preparation used was 80% pure and introduced at a comparable concentration, intravenous injection would need to be 60 to 65 ml. Other routes of delivery such as albumin beads (46) or aerosol need to be considered but would appear to be incapable of providing sufficient concentrations of the protein to the lower tract of the lung.

Practical Considerations and Possible Alternatives

It is likely that replacement therapy with α_1PI would stop the progression of lung disease in nonsmoking individuals with α_1PI deficiency. Such a conclusion was reached by a National Institutes of Health Working Group for the evaluation of elastase inhibitor replacement therapy in pulmonary emphysema held in 1978 in Bethesda, MD (47). The PiM-allele is by far the most common, followed by PiS and PiZ (48). At the present time, most of the severely deficient individuals in the U.S. have not been identified. Mass screening can be achieved, however, and in Sweden all newborn infants are tested routinely (49). In order to assay large populations, a preliminary measurement of α_1PI levels by radial-immunodiffusion should provide an effective cutoff point. Individuals with perhaps 40% or less of the normal levels could then be phenotyped.

Early detection of a deficiency state could provide a powerful motivation to individuals to avoid lung irritants including smoking, working in industrial jobs where there are irritating fumes, dust, smoke, and proteolytic aerosols (e.g., enzyme soaps), and to seek early treatment for respiratory tract infections. Long-term α_1PI therapy could be commenced in deficient individuals during early stages of lung dysfunction. This might be detected by pulmonary function tests including spirometry and the pulmonary diffusing capacity for carbon monoxide (single breath (DL_{CO})) or by chemical tests such as the radioimmunoassay of elastin degradation products, as described in this symposium by Dr. Umberto Kucich.

Estimates of the number of individuals seriously deficient (i.e., < 40% of normal levels) in α_1PI vary. A conservative estimate of 1 in 2,000 to 3,000 individuals in the United States would indicate that perhaps 100,000 to 150,000 individuals are eventual candidates for α_1PI therapy. If perhaps 50% of these individuals could be identified, and if early signs of lung dysfunction are the criteria for replacement therapy, perhaps 25,000 individuals would be potential recipients. In the United States, approximately 3 million liters of plasma yielding 4 million grams of α_1PI are collected yearly (50). With eventual recovery of 40% of this protein from Cohn IV-1 and other plasma fractions, 1.6 million g/year or 32,000 g/ week would be available. Because ~ 4 g/ week are needed per ZZ homozygote to maintain adequate inhibitor levels at all times, a significant fraction of the needs of the community could be met if all α_1PI were used for this purpose.

Of course, the presentation of Dr. Cochrane in the symposium points out the exciting possibility that α_1PI might prove beneficial in controlling ARDS and, if this proves correct, significant supplies of α_1PI would be needed for this purpose. At some point it may become feasible to enhance the supplies of α_1PI through recombinant DNA techniques, and considerable progress has been made in the cloning of α_1PI (51). The large quantities of protein needed/patient as compared with other current projects under way in that industry, however (e.g., growth hormone, insulin, interferon), would suggest an unfavorable cost factor compared with the facile isolation of α_1PI from plasma fractions. In addition, and perhaps even more important, α_1PI is a glycoprotein containing ~ 13% carbohydrate in 3 complex carbohydrate chains. α_1PI produced by recombinant techniques using bacteria or yeast will be either devoid of carbohydrate, or would have modified sugar side chains compared with the normal circulatory inhibitor, and such α_1PI derivatives would need to be evaluated extensively with regard to solubility, activity, clearance rates, antigenicity, etc., prior to clinical use. If these problems can be overcome, however, the reductive-salting out approach to purification should be applicable to an α_1PI product obtained by genetic engineering technology.

Although emphysema in smokers and others exposed to environmental hazards also appears to result from an overwhelming of the antiprotease shield in the lung, the limits of availability of α_1PI probably would place these individuals in a very low priority to receive the drug since other more effective means of intervention (e.g., cessation of smoking) are possible. The eventual introduction of low M.W. synthetic elastase inhibitors might also aid these individuals provided highly specific drugs can be developed that are effective, nontoxic, and long-lasting. This subject is addressed by Dr. James Powers in this symposium.

In conclusion, scientific studies from many laboratories suggest the likelihood that α_1PI can assume a major and beneficial role in preventing emphysema in α_1PI-deficient individuals and may also prove of value in the treatment of ARDS. The industrial production of an active and safe α_1PI product is now achievable, and extensive clinical trials are warranted in the years ahead.

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